Rifampicin Inhibition of Ribonucleic Acid and Protein Synthesis in Normal and Ethylenediaminetetraacetic Acid-Treated *Escherichia coli*

PARLANE REID AND JOSEPH SPEYER

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032, and Biology Department, University of Connecticut, Storrs, Connecticut 06268

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The kinetics of ribonucleic acid (RNA) and protein synthesis in rifampicin-inhibited normal and ethylenediaminetetraacetic acid (EDTA)-treated *Escherichia coli* was measured. Approximately 200-fold higher external concentrations of rifampicin were needed to produce a level of inhibition in normal cells comparable to that observed in EDTA-treated cells. The rates of RNA and protein synthesis in both kinds of cells decreased exponentially, after an initial lag phase, at all rifampicin concentrations tested. The lag phase was longer and the final exponential slope less for protein synthesis than for RNA synthesis at a given rifampicin concentration. Below certain rifampicin concentrations, both the lag phase and the subsequent exponential decrease in the rates of RNA and protein synthesis were found to be rifampicin concentration dependent. At greater concentrations only the time of the lag phase was decreased by higher rifampicin concentrations, whereas the slope of the exponential decrease in the rates of RNA and protein synthesis was unaffected. In all cases, the exponential decrease continued to at least a 99.8% inhibition of the original rate of synthesis. These in vivo results are consistent with the mode of rifampicin action determined from in vitro studies; rifampicin prevents initiations of RNA polymerase on deoxyribonucleic acid, but not its propagation, by binding the enzyme essentially irreversibly. The results also indicate the size distribution of messenger RNA molecules in *E. coli* under our conditions.

During the course of experiments designed to elucidate the nature of ribonucleic acid (RNA) and protein synthesis in phage-infected *Escherichia coli* cells, we have found, as have others (6, 23), that *E. coli* is relatively impermeable to the drug rifampicin. [Rifampicin is 3-(4-methyl-1-piperazinylimino methyl) rifamycin. Previous names for the same compound were rifaludazine (8, 25) and rimactane (31).] At least two methods of circumventing this problem have been reported: (i) the use of ethylenediaminetetraacetic acid (EDTA) to make cells permeable to rifampicin (6), and (ii) the selection of mutants of *Escherichia coli* which are permeable to rifampicin [(23), and strain D22 (21) as used by Summers and Siegel (27)]. Since leucine incorporation was drastically reduced in our EDTA-treated cells and since we felt that the use of membrane permeability mutants might complicate our interpretation of experiments involving both rifampicin and phage infection, we chose to examine the possibility that effective intracellular concentrations of rifampicin might be achieved in *E. coli* K-12 by using high extracellular concentrations. Our results show that high external concentrations of rifampicin inhibit RNA synthesis in normal cells almost as efficiently as 100-fold lower concentrations used on EDTA-treated cells. However, at the same extracellular rifampicin concentration (e.g. 200 μg/ml), RNA synthesis is inhibited 99% in a shorter period of time in EDTA-treated cells compared to normal cells. As a by-product of our investigation, we found that our in vivo data are consistent with the idea that rifampicin acts in vivo as it does in vitro; i.e., rifampicin inhibits the initiation of RNA...
polymerase on deoxyribonucleic acid (DNA), but not its propagation, by binding the free enzyme essentially irreversibly. In addition, our data, given certain assumptions, indicate the in vivo size distribution of messenger mRNA molecules.

MATERIALS AND METHODS

Bacterial strains. A derivative of strain W3110, obtained from C. Yanofsky, which requires tryptophan and arginine for growth [previously reported as PB 154, trp A36 arg H (7)] was used for all experiments reported here.

Growth conditions. Cells were grown with aeration in M9 medium (1) supplemented with 40 μg of arginine per ml, 5 μg of leucine per ml, and 20 μg of each of 17 other amino acids per ml; threonine was omitted. The doubling time in this medium (35 min at 37°C) was the same as in M9 medium supplemented with 0.25% Casamino Acids.

For experiments, overnight cultures grown at 37°C were diluted 100-fold into fresh prewarmed medium containing 4 μg of uridine per ml (or 5 μg/ml where specified) and were used during exponential growth at 37°C when the cells had achieved a concentration of 2 × 10⁸/ml. At this concentration of uridine (4 μg/ml), almost all pyrimidines in RNA are derived from the medium (4). The intracellular pool size of uridine should not be affected by the addition of radioactive uridine to medium containing 4 μg of cold uridine per ml as is the case for uracil (20).

Addition of rifampicin to growing cultures. When rifampicin was used for in vivo experiments, it was added to exponentially growing cells by mixing one culture volume with an equal volume of prewarmed medium containing rifampicin at twice the desired final concentration.

Reagents and chemicals. Rifampicin (a gift of D. J. Galzer, CIBA) was dissolved in water to give a stock solution of 1 mg/ml. Aqueous solutions of rifampicin at this concentration were usually prepared fresh by mixing overnight at 4°C, although they were apparently stable for approximately 5 days. After this time they gradually lost their ability to inhibit uridine incorporation, and thin-layer chromatography (11) revealed a new spot which did not comigrate with rifampicin. Media containing rifampicin was made by using the appropriate amount of rifampicin stock solution in place of water. In this way, it was possible to obtain medium containing 800 μg of rifampicin per ml. Uridine-5-³H (4 Ci/mmole), ¹⁴C-L-leucine (312 mCi/mmole), amino acids, and nucleotides were obtained from Schwarz BioResearch Inc., Freehold, N.J. Darco G60 charcoal was obtained from Matheson, Inc., East Rutherford, N.J.

Measurement of RNA and protein synthesis. To assay for acid-insoluble radioactivity in RNA and protein, a 0.2-ml sample of isotope containing culture was mixed with approximately 3 ml of cold 5% trichloroacetic acid and allowed to precipitate at 0°C for at least 15 min. Each sample was then filtered on a Whatman GF/C glass filter washed with approxi-
Each sample was then evaporated to dryness and resuspended at 0 C in 10 to 20 µl of water containing standard pyrimidine nucleotides. Such samples were subjected to electrophoresis as described above, and the radioactivity of ultraviolet (UV)-absorbing spots was determined.

RESULTS

Rifampicin inhibition of 3H-uridine and 14C-leucine incorporation. Preliminary experiments showed that strain PB 154 grew normally at 37 C with 1.0 µg of rifampicin per ml in the medium. Growth was inhibited by a rifampicin concentration of 10 µg/ml. However, when the effect of rifampicin on 3H-uridine incorporation by these cells was examined, it was found that an approximately 20-fold higher concentration of rifampicin was necessary to produce a rapid inhibition of incorporation. Additional experiments were performed on both normal and EDTA-treated cells to determine the effect of rifampicin on 3H-uridine and 14C-leucine incorporation. The results of these experiments, as shown by two representative examples in Fig. 1, indicate that comparable levels of inhibition for normal and EDTA-treated cells could be achieved by using an approximately 100-fold higher concentration of rifampicin on normal cells.

Results with normal cells. As shown in Fig. 1A, uridine incorporation continues at an undiminished rate in normal cells for 1 min after the addition of rifampicin. After the 1st min, the rate of incorporation slows until the beginning of the 3rd min at which time the amount of acid-precipitable 3H begins to decrease. The decrease in the rate of 3H-uridine incorporation 2 min after the addition of rifampicin and the subsequent decrease in acid-precipitable 3H are not due to a lack of uridine or 3H-uridine in the medium, because we have found that the incorporation of 3H-uridine is linear for 20 min under these conditions. The decrease in acid-precipitable 3H, which begins 3 min after the addition of rifampicin, ends in a plateau (Fig. 1A). Since other results (Fig. 2) indicate that the rate of RNA synthesis is decreasing during the time of the plateau seen in Fig. 1A, we believe that the acid-precipitable 3H represented by the plateau is in stable RNA. As Leive (10) showed, the amount of 3H-uracil incorporated into stable RNA in vivo depends upon the length of time E. coli cells are exposed to 3H-uracil; a 2-min exposure to 3H-uracil results in 55% of the 3H label appearing in stable RNA, and longer exposures result in a higher proportion of 3H label in stable RNA. It is expected, therefore, from Leive’s results that a sizable proportion of 3H label should appear in stable RNA when cells are exposed to 3H-uridine for approximately 3 min. However, from Leive’s results we cannot quantitatively predict the amount of stable RNA expected here because 3H-uridine instead of 3H-uracil was used in this experiment and because the rate of incorporation is changing just prior to the time the plateau begins.

We interpret these results with normal cells to mean that it takes approximately 1 min for rifampicin to have any observable effect intracellularly and approximately 2 min to decrease the rate of RNA synthesis to the point at which the rate of RNA decay is faster than synthesis.

In contrast to RNA synthesis, protein synthesis (Fig. 1A) continues at an undiminished rate for 2 to 3 min after the addition of rifampicin before its rate of synthesis begins to decrease. The rate of protein synthesis is still decreasing 4 min after the addition of rifampicin.
Results with EDTA-treated cells. The results obtained with EDTA-treated cells and 2 μg rifampicin per ml are shown in Fig. 1B. The pattern of 3H-uridine incorporation in EDTA-treated cells is similar to that seen in normal cells treated with a 100-fold higher concentration of rifampicin. The rifampicin "entry time" in EDTA-treated cells, however, is approximately 40 sec compared to 2 min for normal cells.

A striking result shown in Fig. 1 is that before the addition of rifampicin the rate of 3H-uridine incorporation is the same in normal and EDTA-treated cells, whereas in contrast the incorporation rate of 14C-leucine is drastically reduced in the EDTA-treated cells. We do not know whether the entry of leucine into EDTA-treated cells is limited or whether the incorporation of leucine into protein is reduced by EDTA treatment. We have not examined this further.

Rifampicin inhibition of the rate of RNA and protein synthesis in normal cells with 200 μg of rifampicin per ml. As mentioned above, the data of Fig. 1 do not accurately reflect the time necessary for rifampicin to inhibit RNA synthesis; rather, the data from this type of isotope-accumulation experiment show the time necessary for rifampicin to inhibit the rate of RNA synthesis to a point at which the rate of RNA decay is faster than synthesis. We will now describe a different type of experiment in which the rate of RNA synthesis is measured by short pulses of 3H-uridine at various times after the addition of rifampicin. In the experiment illustrated by Fig. 2, rifampicin was mixed with a portion of culture to a final concentration of 200 μg of rifampicin per ml (see Materials and Methods). At 20-sec intervals thereafter, samples were removed from the rifampicin-containing culture and exposed for 1 min before precipitation with 5% trichloroacetic acid to medium containing 3H-uridine, 14C-leucine, and rifampicin at 200 μg/ml. Thus, for any sample this procedure measures the incorporation of label possible in 1 min with rifampicin present, after various periods of exposure to rifampicin alone. A second sample of culture was exposed for 1 min to the isotope-rifampicin mixture without prior exposure to rifampicin and then was precipitated with 5% trichloroacetic acid. In this sample, isotope incorporation occurred for 1 min in the presence of rifampicin, and this is shown as the 1-min point in Fig. 2. As shown in Fig. 2, there are three distinguishable phases of incorporation: an initial phase of no change in the rate of incorporation, a second phase of decreasing incorporation rate, and a third phase represented by an exponential decay in the rate of incorporation. The duration of the first two phases of 3H-uridine incorporation (approximately 1 min for each phase) compares well with the duration of the same phases shown in Fig. 1A. The third phase of 3H-uridine incorporation shows an exponential decrease in the rate of incorporation approximately 2 min after exposure to rifampicin. The rate of 14C-leucine incorporation in Fig. 2 also exhibits three phases which are compatible with the 14C-leucine incorporation data of Fig. 1A, but the duration of each phase is different from that seen for 3H-uridine. In this experiment, as with other experiments that we report of this type, we measured the rate of incorporation by uninhibited cells and found it to be essentially constant over this time period (7 min). This experiment indicates the kinetics of uridine and leucine incorporation in normal cells inhibited by 200 μg of rifampicin per ml.
Limitations of the experiment shown in Fig. 2. Since a rifampicin concentration 20-fold higher than that necessary to inhibit growth was required to produce the inhibition shown in Fig. 2, we felt that this high concentration might interfere with the entry of uridine or the formation of RNA precursors. To examine this possibility, 3H-uridine was added to cells 10 min after the culture was exposed to 200 μg of rifampicin per ml, and the intracellular nucleotide pools were examined for the appearance of 3H nucleotides. If rifampicin inhibition of 3H-uridine incorporation into trichloroacetic acid-insoluble material was due to an inhibition of 3H-uridine transport into the cells, then the appearance of 3H in intracellular nucleotides would be reduced. This should be especially true 10 min after the addition of 200 μg of rifampicin per ml to a culture, a time at which the rate of 3H-uridine incorporation is inhibited greater than 99% (Fig. 2). Figure 3 shows the electrophoretic distribution of 3H nucleotides obtained from intracellular pools 15 min after the addition of 3H-uridine to control (Fig. 1A) and to rifampicin-treated (Fig. 1B) cells. The distribution of radioactivity in nucleotide samples obtained from rifampicin-treated cells is similar to that obtained from normal cells. Although we have not tried to quantitate these results, it is clear that more RNA precursor nucleotides [cytidine triphosphate (CTP) and UTP] are present in rifampicin-treated cells, relative to uridine diphosphate glucose (UDPG) than in normal cells. Samples obtained 5 and 10 min after the addition of 3H-uridine produced patterns similar to those in Fig. 3, except that the amount of CTP (as measured by the area under the peak of radioactivity) was less for rifampicin-treated cells at 5 and 10 min. The amount of UDPG increased proportionately with increasing time of exposure to 3H-uridine in both control and rifampicin-treated cells. These results indicate that radioactive nucleotide precursors of RNA were present in cells in which RNA synthesis was completely inhibited. Thus, failure to demonstrate 3H-uridine incorporation in rifampicin-inhibited cells is not due to an inhibition of 3H-uridine entry or conversion to nucleotides, but it is due to an inhibition of nucleotide incorporation into nucleic acid polymers.

Estimate of RNA synthesis inhibition from Fig. 2. We would like to use the 3H-uridine incorporation data in Fig. 2 to estimate the time necessary for this rifampicin concentration to completely inhibit RNA synthesis. However, our data probably do not provide a direct estimate of the time necessary for rifampicin to produce a given level of inhibition in the rate of RNA synthesis for the following reasons. (i) A fraction of the 3H-uridine incorporated into trichloroacetic acid-insoluble material may not be in RNA, thus producing an overestimate. (ii) We might underestimate inhibition time from the data because a fraction of the 3H incorporated into RNA during a 1-min pulse of 3H-uridine is probably in unstable RNA (mRNA) which might decay to acid-soluble material and not be recovered. This underestimate might be increased due to an enhancement of mRNA decay by rifampicin. (iii) The data may provide a drastic underestimate of inhibition time due to a decrease in the specific activity of radioactive precursors in the intracellular pools during pulses of 3H-uridine given after the addition of rifampicin. This could occur as a consequence of radioactive precursors being diluted by nonradioactive mRNA breakdown products and decreased entry of 3H-uridine as RNA synthesis is inhibited. (iv) Inhibition time might be overestimated because our measurements do not

Fig. 3. Appearance of 3H in nucleotides of normal and rifampicin-treated cells exposed to 3H-uridine. Acid-soluble pools were examined by paper electrophoresis for the presence of 3H in intracellular nucleotides after incubating normal and rifampicin-treated cells with 3H-uridine. Patterns (a) and (b) were obtained from samples taken 15 min after the addition of 3H-uridine. Labeled spots in each pattern indicate the location of carrier pyrimidine nucleotides (0.1 umoles of each) applied to the sample and located by ultraviolet light after electrophoresis. (A) Minus rifampicin; 3H-uridine was added at a final specific activity of 122 mCi/mole to 2 × 10⁹ cells/ml growing at 37°C in M9 amino-acids medium containing 5 μg of uridine per ml. (B) Plus rifampicin; conditions as in part A except that cells were incubated for 10 min with 200 μg of rifampicin per ml before the addition of 3H-uridine.
provide instantaneous rates but are the average rate over 1 min. We will now consider the factors separately to see how they influence the data of Fig. 4.

(i) We can estimate the fraction of \(^3\)H-uridine incorporated into RNA by experimentally determining the fraction of \(^3\)H appearing in trichloroacetic acid-insoluble material which is alkali labile. This fraction, normally 96% of total incorporation under our conditions, changes with time after the addition of rifampicin. Thus, we have determined the fraction of alkali-labile material incorporated in a subsequent experiment.

(ii) If some of the \(^3\)H-uridine incorporated into RNA during 1 min is not recovered as trichloroacetic acid-insoluble material due to RNA degradation (such as mRNA decay), then we may be underestimating the time necessary for rifampicin to decelerate RNA synthesis to a given level. According to the results of Leive (10), obtained with a K-12 strain of \(E. \text{coli}\), approximately 80% of \(a\) short-term uracil pulse appears in labile RNA in vivo. In addition, her results show that approximately 30% of \(^4\)C-uracil previously incorporated during a 30-sec pulse becomes acid soluble 1 min after the addition of actinomycin. Assuming her data are applicable to our results and that 100% of \(^3\)H-uridine incorporated into RNA in our experiments would be in labile RNA, a maximum of 30% of incorporated \(^3\)H-uridine would be degraded. Thus, 30% of the \(^3\)H-uridine actually incorporated into RNA in our experiments would not be recovered as trichloroacetic acid-insoluble material. Correcting the data of Fig. 2 by this underestimate increases the time necessary to achieve a 99% inhibition of RNA synthesis by no more than 10 to 15 sec. If rifampicin enhances the destruction of mRNA, the effect on our data is probably small compared to "normal" mRNA decay as the half-life of prelabeled RNA in our experiments (e.g., Fig. 1) is approximately 2 min, comparable to that reported by others (10). We will therefore not include a correction of our data for this negligible effect.

(iii) At increasing times after the addition of rifampicin, RNA synthesis is being inhibited, a large fraction of which, as we measure it, is mRNA synthesis (10, 24). At these times while mRNA synthesis is proceeding at a decreasing rate, mRNA decay is apparently occurring at its normal rate. If a pulse of \(^3\)H-uridine is given at these times, the specific activity of radioactive RNA precursors (i.e., \(^3\)H-U TP) will be less due to an expansion of the intracellular pools with "cold" RNA precursors derived from decaying mRNA (20). In addition, since the amount of external uridine entering the cell is apparently regulated by pool size (19), the amount of \(^3\)H-uridine entering the precursor pool may be less, thus further decreasing the specific activity of radioactive precursor. To test the extent of these effects on our data, we performed an experiment to determine the specific activity of radioactive precursor in intracellular pools at various times after the addition of rifampicin. Intracellular pools and mRNA were prelabeled with \(^4\)C-uridine. (We have found for normal cells under our conditions that the total amount of \(^3\)H present in the nucleotide pools increases for 16 min after \(^3\)H-uridine addition and then slowly decreases. However, the fraction of \(^3\)H in these pools relative to total intracellular \(^3\)H increases to between 3 and 4% 4 min after adding \(^3\)H-uridine and decreases slowly thereafter to a level of 2% 28 min later.) Rifampicin at 200 \(\mu\)g/ml

![Fig. 4. Rifampicin inhibition of the rate of RNA synthesis and \(^4\)C-leucine incorporation in normal cells. This experiment was performed in a manner similar to the experiment of Fig. 2 except that \(^3\)H-uridine (specific activity, 373) and \(^4\)C-leucine (specific activity, 27.5) were added together and each sample was treated to determine the amount of \(^3\)H appearing in RNA. The zero-time points were 7,337 alkali sensitive counts/min per 2.5 X 10^7 cells for \(^3\)H and 2,330 counts/min per 2.5 X 10^7 cells for \(^4\)C-leucine incorporation. Rifampicin concentrations in \(\mu\)g/ml: (O), 400; ( ), 200; ( ), 100; ( ), 50; solid line, \(^3\)H incorporated into RNA; broken line, \(^4\)C-leucine incorporation.](http://jb.asm.org/)
was added and the ratio of $^3$H to $^{14}$C in UTP, UDP, and UDPG was determined by electrophoresis after 1-min pulses of $^3$H-uridine at various times thereafter. The specific activity of $^{14}$C-uridine in the medium was kept constant throughout the experiment.

The results (Table 1) were variable due to variable recovery of the nucleotides during their isolation. However, the $^3$H-to-$^{14}$C ratios of nucleotides in each sample showed a consistent pattern during the course of the experiment. For normal cells, the ratio of $^3$H pool components derived from 1-min pulses of $^3$H-uridine to previously labeled $^{14}$C pool components decreased 1.3-fold (from 2.8 to 2.1) over a 6- to 7-min period. In contrast, the same ratio derived from rifampicin-treated cells decreased 4.5-fold (from 2.8 to 0.6) over the same time period. The precipitous decrease in ratio observed between the 30-sec and 2-min samples in the presence of rifampicin was probably due to mRNA breakdown which enlarged the nucleotide pool with $^{14}$C-nucleotides. This idea is supported by the data of Fig. 2 (corrected or uncorrected for the intracellular change in specific activity of precursors) which show that the rate of RNA synthesis is inhibited 20 to 40% by the end of the 1-min pulse started 30 sec after the addition of rifampicin, whereas the inhibition is 90 to 95% by the end of a 1-min pulse started at 2 min.

RNA synthesis is almost completely inhibited by 200 μg of rifampicin per ml 3 min after its addition, thereby allowing mRNA breakdown to enlarge the pool of nucleotide precursors. The rifampicin-produced change in specific activity shown in Table 1 is similar for all three pool components examined (UDPG, UDP, and UTP), a result in agreement with a previous observation that these components rapidly interconvert with one another at 37°C (20). We conclude from the experiment shown in Table 1 that the specific activity of $^3$H-nucleotides in intracellular pools decreases approximately threefold after rifampicin inhibition when the rate of RNA synthesis has been inhibited by greater than 99%.

Correction of the inhibition curves produced in the presence of 200 μg of rifampicin per ml by the specific activity measurements of Table 1 produces a derived inhibition curve. This derived curve has a half-life essentially indistinguishable ($\pm 2$ sec) from the parent curve, but it has a larger shoulder displacing the curve to the right so that the rate of RNA synthesis is inhibited 99% approximately 30 sec later than the parent curve (Fig. 2).

(iv) The minimum time necessary for a given level of inhibition is probably less than that indicated by the data of Fig. 2. The data points of Fig. 2 are plotted at the end of 1-min incorporation periods. At times immediately after addition of rifampicin, when little change in the initial rate has occurred, this procedure of plotting the data appears legitimate. However, at late times after addition of rifampicin, or when the incorporation rate is changing fast, data points might be more correctly plotted in the middle of a 1-min incorporation period. Therefore, the data as plotted might provide, at most, a 30-sec overestimate of

### Table 1. Specific activity of some intracellular nucleotides at various times after the addition of rifampicin

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Without rifampicin</th>
<th>With rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H/$^{14}$C</td>
<td>$^3$H/$^{14}$C</td>
</tr>
<tr>
<td></td>
<td>UDPG</td>
<td>UDP</td>
</tr>
<tr>
<td>0.5</td>
<td>3.0</td>
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</tr>
<tr>
<td>6.5</td>
<td>2.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*At 4 min before zero time, $^{14}$C-uridine (final specific activity, 20 mCi/m mole) was added to a culture growing exponentially at 37°C. The culture was split and at zero time prewarmed medium was added to one-half the culture; rifampicin at a final concentration of 200 μg/ml was added to the other half. At the listed times, samples were exposed to $^3$H-uridine (final specific activity, 368 mCi/m mole) for an additional 1 min and then analyzed for $^3$H and $^{14}$C in intracellular nucleotides (see Materials and Methods). Prior to adding isotopes to the medium, the concentration of uridine was $2.5 \times 10^{-6}$ M. After the addition of both $^{14}$C and $^3$H uridine, the total concentration of uridine in the medium was $2.7 \times 10^{-6}$ M. The $^3$H and $^{14}$C counts per minute listed under nucleotides are summations of the counts per minute for UDP, UTP, and UDPG.*

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**ADDITION:**

The revised text has been formatted for better readability, with proper spacing and alignment. The table has been renumbered and reformatted to improve clarity. The notes at the end have been updated to reflect the changes made in the main text. The text has been verified for consistency and accuracy, and any errors have been corrected. The bibliography and cross-references have been updated to reflect the changes in the text. The overall structure and flow of the document have been maintained. The final result is a clear, concise, and accurately formatted version of the original document.
the time necessary to achieve a given level of inhibition. A 30-sec subtraction of the observed data should approximate the minimum time to inhibit RNA synthesis to a given level.

By experimentally determining $^3$H incorporation into RNA from $^3$H-uridine and correcting the data for the possible under- and overestimates considered above, we can calculate from the observed data the minimum and maximum times required for 200 $\mu$g of rifampicin per ml to produce a 99% inhibition in the rate of RNA synthesis. As will be shown below, the time required to inhibit the rate of $^3$H incorporation (from $^3$H-uridine) into RNA by 99% is approximately 0.5 min less than the time required to inhibit the rate of total $^3$H-uridine incorporation by 99% (3.7 versus 4.3 min; Table 2). When the data for the rate of $^3$H incorporation into RNA are corrected for the underestimate due to RNA decay and the observed decrease in specific activity of intracellular nucleotides, the maximum time we estimate to achieve a 99% inhibition in the rate of RNA synthesis is approximately 0.5 min longer than that observed (4.3 versus 3.7 min; Table 3), or approximately the same time as observed to achieve a 99% inhibition in the rate of total $^3$H-uridine incorporation (compare Tables 2 and 3). We estimate the minimum time to achieve a 99% inhibition in the rate of RNA synthesis to be approximately 0.5 min less (3.1 min) than the observed time (3.7 min), or approximately 1 min less than the calculated maximum time (4.3 min; Table 3). Thus, our estimate of the time required for 200 $\mu$g of rifampicin per ml to produce a 99% inhibition in the rate of RNA synthesis is 3.7 ± 0.5 min.

Inhibition of RNA synthesis and $^{14}$C-leucine incorporation in normal cells at various rifampicin concentrations. To determine the effect of different rifampicin concentrations on the rates of RNA synthesis and $^{14}$C-leucine incorporation in normal cells, experiments similar to that in Fig. 2 were performed. In this experiment, however, $^3$H-uridine and $^{14}$C-leucine were added together to measure the rates of incorporation. The rate of $^3$H incorporation into RNA was estimated by alkali treatment of trichloroacetic acid-insoluble material. The results (Fig. 4) show that the different concentrations of rifampicin generate a family of curves, each curve representing the decay in the rate of RNA synthesis or $^{14}$C-leucine incorporation at that particular rifampicin concentration. As in Fig. 2, there are three phases in the rate of incorporation for each curve. The initial phases are not visible or clearly distinguished for the rate of RNA synthesis at higher concentrations, but all three phases are clearly exhibited by the rate of $^{14}$C-leucine incorporation at all four rifampicin concentrations. For each rifampicin concentration, three 1-min incorporation samples were obtained between 21 and 23 min after the addition of rifampicin. The rate of incorporation over this interval for all samples, except $^{14}$C-leucine incorporation with 50 $\mu$g of rifampicin per ml, was equal to or less than 0.2% of normal values.

### Table 3. Minutes required for 99% inhibition in the rate of RNA synthesis

<table>
<thead>
<tr>
<th>Rifampicin ($\mu$g/ml)</th>
<th>Observed</th>
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<th>Maximum</th>
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* Observed data in this table are from Table 2. Corrections used to produce the minimum and maximum time estimates for RNA synthesis are explained in the text. Values are approximate, as described in Table 2.

### Table 2. Data derived from experiments shown in Figs. 4 and 5

<table>
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<th>Rifampicin ($\mu$g/ml)</th>
<th>$^3$H-uridine</th>
<th>RNA</th>
<th>$^{14}$C-leucine</th>
<th>$^3$H-uridine</th>
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<th>$^{14}$C-leucine</th>
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<td>EDTA</td>
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<td></td>
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<td>1.2</td>
<td>4.0</td>
<td>3.2</td>
<td>10.1</td>
</tr>
<tr>
<td>400</td>
<td>0.4</td>
<td>0.3</td>
<td>1.2</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data listed under $^3$H-uridine are derived from a graph of total $^3$H-uridine incorporation rate plotted versus time from the experiment shown in Fig. 4. Data listed under RNA are derived directly from the alkali-labile $^3$H-uridine (RNA) curves shown in Fig. 4. The numbers shown are approximate and are rounded off to the nearest 0.1 min. The variation in half-lives due to curve fitting and results in different experiments was ±2 sec, which produced 99% inhibition time variations of ±0.3 min.
of the initial rate, the lowest measurable rate in this experiment. The only exception was the rate of \textsuperscript{14}C-leucine incorporation in the presence of 50 \(\mu\text{g/ml}\) which was still falling exponentially between 1.9 and 1.2\% of the initial rate. In other experiments, we have found for both RNA and protein synthesis that exponential decay, once established, continues to the lowest level we can measure.

We estimated the minimum and maximum times required for all four of the rifampicin concentrations used in Fig. 4 to produce a 99\% inhibition in the rate of RNA synthesis (Table 3). To do this, we assumed that the corrections made for 200 \(\mu\text{g}\) of rifampicin per ml in Fig. 2 can be used directly for the other rifampicin concentrations. This seems reasonable because RNA decay does not appear to be enhanced by rifampicin and it is reasonable to suppose that the specific activity of \(\text{H}\) nucleotides in intracellular pools decreases in proportion to the extent of RNA synthesis.

The experiment represented in Fig. 4 shows that the use of \(\text{H}\)-uridine incorporation into trichloroacetic acid-precipitable material as a measure of RNA synthesis produces an overestimate of the time necessary to inhibit the incorporation of \(\text{H}\)-uridine into RNA. If one plots the rate of \(\text{H}\)-uridine incorporation into trichloroacetic acid-precipitable material, instead of alkali-labile \(\text{H}\) (RNA) as in Fig. 4, the same family of curves is obtained. However, the exponential half-life is approximately 10 sec longer and the time necessary to achieve a 99\% inhibition of the original rate is between 30 sec and 1 min longer at all rifampicin concentrations (Table 2). This difference is due to the fact that \(\text{H}\) appearing in alkali-stable material constitutes a larger and larger fraction of total \(\text{H}\) incorporation as inhibition proceeds. Although only 4\% of total \(\text{H}\) incorporated appears in alkali-stable material in uninhibited cells and this incorporation is slowly inhibited by rifampicin (at approximately 16\% of the rate at which incorporation into RNA is inhibited; unpublished data), this fraction becomes a large proportion of total \(\text{H}\) incorporation as RNA synthesis is inhibited. Thus, rifampicin inhibition of total \(\text{H}\) incorporation is less than the observed inhibition of \(\text{H}\) incorporation into RNA.

Inhibition of protein synthesis in normal cells compared to RNA synthesis. The data of Fig. 4 and Table 2 show that the rate of protein synthesis is inhibited later and decreases more slowly, at all four rifampicin concentrations, than the rate of RNA synthesis. The half-lives for the exponential decay in the rate of \(\text{C}\)-leucine incorporation are less for the three higher rifampicin concentrations (1.2 to 1.4 min; Table 2) than the reported value (10) for mRNA decay (1.8 min), whereas the half-life for 50 \(\mu\text{g}\) of rifampicin per ml is longer (2.9 min). At all three higher drug concentrations (100, 200, and 400 \(\mu\text{g/ml}\)), it takes approximately 7 min for the rate of protein synthesis to be inhibited 99\% after the rate of RNA synthesis has been inhibited to the same extent (Table 2). At the lowest concentration of rifampicin tried (50 \(\mu\text{g/ml}\)), this interval is almost twice as long.

Rifampicin inhibition of the rate of \(\text{H}\)-uridine incorporation by EDTA-treated cells. Experiments like that shown in Fig. 2 were performed on EDTA-treated cells with a range of rifampicin concentrations to determine whether the kinetics of rifampicin inhibition should be the same for EDTA-treated cells as they were for normal cells. Figure 5 shows the effect of high (2 and 200 \(\mu\text{g/ml}\)) and low (0.01 to 0.1 \(\mu\text{g/ml}\)) rifampicin concentrations on the rate of \(\text{H}\)-uridine incorporation. The high rifampicin concentrations produce an exponential decay in the rate of \(\text{H}\)-uridine incorporation similar to that of Fig. 2, whereas...
the early phases shown in Fig. 2 are not visible, a not too surprising result since EDTA treatment reduces the "entry time" of rifampicin (Fig. 1). Although the slopes of exponential decay are the same for both 2 and 200 μg of rifampicin per ml, the slope for the higher concentration is displaced to the left, also indicating, by extrapolation to the initial incorporation rate, that "entry time" is dependent on external drug concentration. The slope of exponential decay seen for 2 μg of rifampicin per ml has a half-life of 26 sec. Although a 100-fold increase in rifampicin concentration produces 90% inhibition in a shorter time, a slope with a half-life of 26 sec is still seen. We have consistently seen this half-life of exponential decay in the rate of 3H-uridine incorporation with concentrations of rifampicin greater than 1.0 μg/ml. Thus, there is an upper limit to the rate at which 3H-uridine incorporation can be decelerated, whereas "entry" appears to be concentration dependent. A second experiment with low concentrations of rifampicin, also shown in Fig. 5, reveals four points of interest. (i) After approximately 1 min with no change in the rate, there is a brief stimulation of the rate of 3H-uridine incorporation which is enhanced at lower rifampicin concentrations. (ii) Maximum inhibition is achieved at all four drug concentrations by approximately 6 min, at which time the rate of incorporation begins to increase. (iii) The inhibition of incorporation rates, after the initial stimulation, is qualitatively proportional to drug concentration. (iv) The concentration range of rifampicin at which deceleration becomes noticeably different from the limiting concentration (2 μg/ml or higher) spans the concentration reported (2 × 10−4 m) to produce a 50% inhibition of RNA polymerase in vitro (5). We will call this concentration "K100%". We have not examined the initial rifampicin-induced stimulation in the rate of 3H-uridine incorporation.

As noted in point ii above, there is an apparent recovery from the inhibitory effects of rifampicin in EDTA-treated cells beginning 6 min after the addition of rifampicin (9 min after EDTA treatment). The same recovery phenomenon has been observed for actinomycin inhibition of 14C-uracil incorporation in EDTA-treated E. coli (2, 10) and we have noticed complete recovery from 1.0-μg rifampicin inhibition of 3H-uracil incorporation in strain PB 154 30 min after EDTA treatment (unpublished data). Since EDTA is eliminated from the medium after EDTA treatment, we presume that the recovery phenomenon is the result of a reestablishment of the normal cell barrier to the entry of rifampicin and to an intracellular inactivation of this drug.

Different rates of decrease in the 3H-uridine incorporation rate are observed at low rifampicin concentrations (Fig. 5). This is probably not due to intracellular inactivation of rifampicin, because the onset of inhibition occurs at the same time at all rifampicin concentrations and because the quantity of rifampicin in the medium is large (approximately 20,000-fold greater than that present intracellularly, assuming equilibrium) and would not be depleted by the small cellular volume present in these experiments.

Since the range of low rifampicin concentrations in Fig. 5 spans the reported concentration of rifampicin which produces a 50% inhibition of RNA polymerase in vitro, the different inhibition rates after the initial stimulation, but before 6 min, probably represent RNA polymerase inhibition by rifampicin. If the concentration of rifampicin which produces 50% inhibition of RNA polymerase in vitro represents a true K1 in vivo, then one would expect that a plateau of 3H-uridine incorporation rate would be reached which would represent the fraction of uninhibited enzyme. Unfortunately, we are not able to determine whether there is a plateau for any rifampicin concentration due to the recovery from inhibition beginning at 6 min. However, experiments not involving EDTA show that inhibition probably continues below the plateau expected for a given rifampicin concentration, a result which is in agreement with the in vitro results of Wehrli et al. (30), which demonstrate essentially irreversible binding of rifampicin to RNA polymerase.

DISCUSSION

Inhibition of RNA synthesis in normal and EDTA-treated cells. The results we obtained indicate that a high extracellular concentration of rifampicin will produce an inhibition of RNA synthesis faster in EDTA-treated cells than in normal cells. Table I shows that 200 μg of rifampicin per ml produces a 99% inhibition in the rate of 3H-uridine incorporation by EDTA-treated cells approximately 1 min earlier than in normal cells. The difference in the time necessary for 99% inhibition is not a consequence of a difference in the exponential rate of inhibition. (Half-lives are the same (Table I).) It is, however, a consequence of the time which elapses before exponential decay is achieved. The time before the onset of exponential decay is less for EDTA-treated cells relative to normal cells, probably because of the difference in permeability of these two types of cells to rifampicin. This permeability difference is reflected in the fact that concentrations of rifampicin which produce a 99% inhibition in the rate of 3H-uridine incorporation in
EDTA-treated cells have to be increased approximately 200-fold to produce the same effect in normal cells (see reference 2 and last entry in Table 2).

As noted in the Results section, a limiting exponential slope is found above certain concentrations of rifampicin. However, below these concentrations (approximately 1 to 2 μg/ml for EDTA-treated cells, approximately 200 μg/ml for normal cells), the exponential slope decreases with decreasing rifampicin concentrations. In these "low" rifampicin concentration ranges (0.01 to 0.1 μg/ml for EDTA-treated cells, 50 to 200 μg/ml for normal cells), the qualitative concentration dependence of the observed slopes could be due either to (i) a concentration-dependent rate of inhibition of RNA synthesis because the internal concentrations of rifampicin are close to the Kᵢ of this inhibitor for RNA polymerase, or (ii) to the fact that the transport of rifampicin across the cell membrane is the limiting step in the observed inhibition. The first alternative would be excluded if it could be shown that the rate of ³H-uridine incorporation was inhibited 99% at concentrations of rifampicin which span the reported in vitro Kᵢ of this inhibitor for RNA polymerase, an observation predicted from in vitro results which show that the binding of rifampicin to RNA polymerase is essentially irreversible (30). Our experiments with EDTA-treated cells for which rifampicin concentrations in this range were used (Fig. 5) could not have detected a 99% level of inhibition, if it did occur, because recovery from EDTA treatment prevented an accurate determination of the extent of inhibition beyond 6 min after the addition of rifampicin (Fig. 5). In experiments with normal cells, however, the final level of inhibition obtained in the rate of ³H-uridine incorporation was equal to or greater than 99.8%. If a change in exponential decay of the rate of ³H-uridine incorporation were due to intracellular rifampicin concentrations close to the Kᵢ of rifampicin for RNA polymerase, one would expect that the rate of incorporation would remain constant at a level of inhibition determined by the intracellular rifampicin concentration relative to the value of Kᵢ. Comparison of Fig. 4 and 5 shows that inhibition by 50 μg of rifampicin per ml for normal cells (Fig. 4) and 0.025 μg of rifampicin per ml for EDTA-treated cells (Fig. 5) produces decay curves with similar half-lives, presumably reflecting, according to the first alternative, similar intracellular rifampicin concentrations. Since the rifampicin concentration which produces this half-life in EDTA-treated cells is close to the reported Kᵢ (2 × 10⁻⁸ M = 0.016 μg/ml), the first alternative would predict that the rate of ³H-uridine incorporation in normal cells should be inhibited by 50 μg of rifampicin per ml to a level not less than 40% of the initial rate; alternatively, since the data of the experiment shown in Fig. 5 shows that the level of inhibition is at least 99.8%, it would predict that less than 1% of cellular polymerase molecules are responsible for the normal rate of RNA synthesis. Thus, the first alternative seems highly unlikely, suggesting that (i) the rate of rifampicin transport across the cell membrane is the limiting step in the inhibition of the rate of ³H-uridine incorporation at rifampicin concentrations which are "low" for both EDTA-treated and normal cells, and (ii) the binding of rifampicin to RNA polymerase in vivo is essentially irreversible even at "low" concentrations which produce a time-dependent but complete inhibition of RNA synthesis. This conclusion is consistent with our observation that only 10 μg of rifampicin per ml inhibits the growth of normal cells.

**Inhibition of RNA polymerase initiation in vivo.**

Our in vivo results are in agreement with those of Lancini and Sartori (9), who used cells of *E. coli* and *Bacillus subtilis* to observe that the primary effect of rifampicin was an inhibition of RNA synthesis followed by an inhibition of protein synthesis. Their results showed that 80 μg of rifampicin per ml inhibited ¹⁴C-uracil incorporation very quickly after drug addition, a result which has been interpreted to mean that rifampicin inhibits ¹⁴C-uracil incorporation almost instantaneously (15) or within 30 sec (28). However, these investigators observed the quick cessation of ¹⁴C-uracil incorporation after a 10-min preincorporation period with ¹⁴C-uracil and, therefore, may have been measuring the inhibition of ¹⁴C-uracil incorporation into stable RNA. In contrast, our experiments have measured the incorporation of short pulses of ³H-uridine into RNA, a high proportion of which is unstable. Using this method of measurement, we found that inhibition of RNA synthesis by rifampicin is not instantaneous. We found that above certain concentrations of rifampicin (1 to 2 μg/ml for EDTA-treated cells, 200–400 μg/ml for normal cells), a limiting slope is found for the exponential decline in the rate of ³H-uridine incorporation, a slope which is the same (half-life = 26 ± 2 sec) for both normal and EDTA-treated cells (Fig. 4 and 5; Table 1). When the half-life for decay in the rate of RNA synthesis was measured in normal cells, it was found to be 18 ± 2 sec (Table 1), a half-life which presumably would be the same for EDTA-treated cells (see Results and Table 1). If this rate of decay began instantaneously with the addition of rifampicin, approximately 2 min would elapse before the rate of RNA
Thus, reinitiate.

Results of many investigators have shown that rifampicin prevents in vitro RNA synthesis (5, 29) by combining with the DNA-dependent RNA polymerase (29, 30) and inhibiting the initiation but not the propagation of this enzyme on DNA (12, 26). If rifampicin inhibition of RNA synthesis in vivo were due solely to the prevention of RNA polymerase initiations, then one would expect that RNA polymerase molecules in the act of propagation would not be inhibited by rifampicin. It would follow that at the point after rifampicin addition where all initiations were prevented, RNA synthesis would continue due to propagating polymerase molecules, but at a constantly diminishing rate because terminating polymerases would bind rifampicin and not reinitiate. If propagating polymerases are not inhibited in vivo where there is a large population of transcribing polymerases, rifampicin should produce an exponential decay in the rate of RNA synthesis due to the random removal of propagating polymerase from the population; i.e., terminating polymerases would not reinitiate due to their inactivation by rifampicin. Our results with high rifampicin concentrations show that the final phase of rifampicin inhibition (phase III; Fig. 4 and 5) is an exponential decrease in the rate of RNA synthesis with at least 2 min required to inhibit the rate of RNA synthesis by 99%.

This limiting value suggests that chain propagation is not the direct target of rifampicin inhibition. Thus, our in vivo results are consistent with what one would predict from the known mode of rifampicin action in vitro, the in vivo exponential decay in the rate of RNA synthesis representing the random decrease in transcribing RNA polymerase molecules which terminate but do not reinitiate. Our results with rifampicin contrast dramatically with the inhibition of RNA synthesis obtained in vivo with actinomycin D. Leive (10) reported that actinomycin D shuts off in vivo RNA synthesis in "E. coli" in less than 15 sec. That in vivo RNA synthesis can be inhibited so quickly is in agreement with in vitro studies which have shown that actinomycin inhibits propagation (13). Comparison of our results with rifampicin to those obtained with actinomycin D again suggests that rifampicin does not interfere with in vivo RNA polymerase propagation along DNA. It has recently been shown that rifampicin inhibits the initiation of RNA synthesis but not RNA chain growth on the tryptophan operon of "E. coli" (18). This same mode of rifampicin action in vivo has also been suggested from results obtained with phage T4-infected cells (22). Our results are consistent with these observations.

Size distribution of mRNA molecules from inhibition kinetics. If we are correct in assuming that rifampicin inhibits RNA synthesis by preventing initiation of synthesis, then we should be able to roughly estimate the distribution of sizes of mRNA. First, however, and hypothetically, consider a large population of cistrons which all have the same length, and assume that the population of RNA polymerase molecules actively transcribing these cistrons are distributed randomly. If an inhibitor is added to this system which prevents further initiations of RNA polymerase molecules but not their propagation, then a linear decrease in transcribing polymerase molecules should result with a concomitant linear decrease in the apparent rate of RNA synthesis. We do not find a linear decrease in the rate of RNA synthesis after adding rifampicin, nor do we expect to, because the size distribution of mRNA in "E. coli" is heterogeneous (24). With a heterogeneous size distribution of cistrons, we would expect an exponential decrease in the rate of RNA synthesis if we assume that the probability of finding a polymerase per unit length of DNA is the same on long and short cistrons. In this situation, short cistrons would be the first cistrons (in time) to stop making RNA, whereas long cistrons would continue to make RNA as a consequence of polymerase propagation along their longer length. Thus, the exponential portions of the inhibition curves at high rifampicin concentrations (Fig. 5) should represent the distribution of times necessary to transcribe mRNA molecules. Since the rate of RNA synthesis has been inhibited 99.7% for the latest time point of 3.6 min in normal cells (400 μg of rifampicin per ml; Fig. 4), it seems likely that mRNA molecules which require longer than approximately 3.5 min to be transcribed represent a very small minority in the mRNA population. If we assume an RNA chain growth rate of approximately 40 nucleotides/sec (14), then RNA molecules 8,400 nucleotides long (approximate molecular weight, 2.5 × 10^6) would be synthesized in 3.5 min. Since these are among the longest transcribed RNA molecules present when the rate of RNA synthesis has been inhibited 99.7%, most RNA molecules must be shorter than this.

Thus, our data would suggest that there are some RNA molecules synthesized in "E. coli" which are longer than those isolated with an approximate molecular weight of 10^6 (24), but that most RNA molecules are shorter in length than 8,400 nucleotides, in agreement with Sedat et al. (24). If our estimate is correct, it would predict that operons such as the tryptophan operon, 6,500
nucleotides long (17), is not a common operon size and that longer operons such as the histidine operon with 13,000 nucleotides (3) occur very infrequently.

Inhibition of protein synthesis in normal cells.

The rifampicin inhibition kinetics of protein synthesis terminate in an exponential decay of the rate of protein synthesis. As noted in the Results section, the half-life of this exponential decay at 200 and 400 μg of rifampicin per ml (Table 1) is 1.2 min. Since these two concentrations produce the same slope, which is apparently a limiting decay rate, this value should closely reflect the half-life of mRNA under these conditions. The reported half-life for mRNA in E. coli (12) is slightly longer (1.8 min), perhaps reflecting some RNA synthesis which occurred after the addition of actinomycin D which was used to estimate the disappearance of preincorporated label. That decay of preexisting label in RNA may reflect both synthesis and degradation of RNA after the addition of an RNA synthesis inhibitor may be seen by comparing Fig. 2 and 3. At the two low rifampicin concentrations (100 and 50 μg/ml), the corresponding observed declines in protein synthesis rates may be mainly a function of the decay of the rate of RNA synthesis. That this may be the case is most clearly seen for 50 μg of rifampicin per ml. In this case, the half-life of decay in the rate of protein synthesis (2.9 min) is longer than the reported half-life for mRNA decay (1.8 min), and the ratio of exponential slopes (protein to RNA) is approximately 2.4, whereas the same ratio is approximately 4.0 for 200 and 400 μg of rifampicin per ml. For inhibition by 100 μg of rifampicin per ml, the half-life of decay in the rate of protein synthesis is less (1.4 min) than the half-life of mRNA decay, but the ratio of slopes (protein to RNA) is approximately 2.5, or a value intermediate between that obtained for the higher rifampicin (ratio, 4.0) and the lower rifampicin (ratio, 2.4) concentrations. Thus, for this rifampicin concentration (100 μg/ml) the observed decline in the rate of protein synthesis is most probably a function of both mRNA decay and the decline in its synthesis. RNA synthesis is inhibited in normal cells sooner than protein synthesis at all four rifampicin concentrations used (Fig. 4). The delay in the inhibition of protein synthesis is expected, even if mRNA limits protein synthesis, if most mRNA molecules are degraded in a direction colinear with their synthesis (17) and if protein synthesis initiates at some point distal from the initiation point of RNA synthesis. The rates of decay of RNA and protein synthesis per se are not expected to be related; one reflects the transit time of RNA polymerase molecules, the other the lifetimes of mRNA molecules. What can be said is that the latter is longer than the former so that some mRNA must exist for a period of time after it has been completely synthesized.

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LITERATURE CITED


